EVALUATION OF SYNERGISTIC AND ANTIMICROBIAL POTENTIAL OF SELECTED HERBS AGAINST ISOLATES OF NEARBY AREA OF HOSPITALS FROM HAMIRPUR, HIMACHAL PRADESH

SYNOPSIS

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INTRODUCTION

India is known for its plant diversity. Due to the climate of India, a wide variety of plants and vegetation is present in the different geographical areas. A large percentage, around 75% of India's population, lives in the villages near the forest area. Medicinal plants are an important part of ancient medicine and modern medicine. The use of medicinal plants in the manufacture of traditional medicine to treat disease is known as (LHT) Local Health Tradition (Ansari and khan, 2018). As we know, plants are enriched in phytochemicals such as alkaloids, quinines and terpenoids. They also synthesize aromatic compounds such as phenols and their oxygen derivatives. Compared to other countries in the world, India is one of the main users of these plants for home remedies to treat various diseases.

Hamirpur is a small district of Himachal Pradesh but contains many medicinal plants that are of great importance in medicine and also have religious significance. The majority of the population here lives in the villages and they have a great knowledge of the traditional methods of using medicinal plants and local herbs for their own benefit. Herb and plant extracts are the basis of Ayurvedic medicine. A lot of work has been done in this field since ancient times, and it takes a lot of effort to explore this treasure for the benefit of mankind. Infectious diseases are responsible for almost half of deaths in equatorial countries with hot and humid climates (example India). In developed countries, despite their understanding of microorganism-caused infections and disease control, incidents such as epidemics are common and may occur due to neglect of precautions or drug resistance, and cause many public health concerns (Iwu *et al.*, 1999).

Many plants contain various bioactive compounds that are observed to have antimicrobial activity against a broad spectrum of microorganisms including bacteria, fungi and viruses. Herbal antimicrobials represent a huge untapped resource for medicine, they have tremendous therapeutic potential as they can serve their purpose without the side effects often associated with synthetic antimicrobials (Hussain and Gorsi, 2004). The irregular, extensive and inappropriate use of the antibiotic leads to antimicrobial resistance in the microorganisms and this is an emerging problem in medical science. Therefore, there is an increasing demand for the development of new antimicrobial agents capable of reducing the use of antibiotics and also ignoring the developed resistance (Natalia *et al.*, 2021).

It is observed that the rhizosphere flora of medicinal plants is of great importance. These microorganisms produce some factors that protect medicinal plants from pathogens and have antimicrobial properties used in the production of antibiotics. The soil is the source of a rich number of microorganisms. It consists of natural as well as one of the most complex habitats for microorganisms. Bacteria are the most common microorganisms found in soil, after which fungi occupy the second position. Their occurrence depends on the chemical and physical parameters of the soil (Pandey *et al.*, 2020). A large amount of waste is present in landfill areas, which serves as a food source for the growth of microorganisms. Microorganisms grow on these wastes, decompose them and utilize the decomposed compounds. Due to their occurrence in larger quantities, there is a greater risk of infection, and mutations can also occur, leading to resistance of microorganisms to various drugs.

Hospital waste and effluent are unwanted by-products of medical development. Healthcare waste contains a large number of potentially harmful microorganisms. These microorganisms are responsible for causing various diseases in hospital patients, healthcare workers and the general public. The disease can potentially be transmitted from an infected person to a healthy person through various factors such as air, soil and water. If not handled properly and disposed of openly, hospital waste can endanger both the environment and human health. Living near these hazardous waste dumps can lead to gastrointestinal infections, lung infections, and reproductive disorders.

Soil-borne diseases pose significant threats to agricultural productivity, impacting crop yield, quality, and overall sustainability. Caused by pathogenic microorganisms dwelling in the soil, these diseases can lead to devastating consequences for global food security and environmental stability. Conventional approaches to disease management, such as chemical pesticides and fumigation, have been instrumental in curbing outbreaks. However, their long-term effects on soil health and the potential risks to human health raise concerns about their sustainability. It is in this context that the integration of medicinal plants into disease management strategies holds immense potential.

The intricate relationship between soil and microorganisms serves as the foundation for understanding the mechanisms underlying the efficacy of medicinal plants against soil-borne diseases. Within the soil ecosystem, a diverse array of beneficial microbes, including bacteria, fungi, and actinomycetes, play crucial roles in nutrient cycling,

disease suppression, and plant growth promotion. Harnessing the power of these microbial allies, medicinal plants possess a unique ability to modulate soil microbial communities, fostering a balance that can impede the proliferation of soil-borne pathogens.

Drawing upon the knowledge amassed from traditional systems of medicine, which have long relied on the healing properties of various plant species, the utilization of medicinal plants holds promise for developing sustainable and environmentally friendly solutions. By exploring the multifaceted interactions between soil, microbes, and traditional herbal remedies, we can tap into nature's wisdom to unlock novel approaches for managing soil-borne diseases while promoting the overall health and resilience of agro ecosystems

According to the WHO, herbal ingredients are the best source for a variety of medicines, as a large number of herbs are recognized as a natural source of antimicrobial agents and represent a best alternative to use in treating the problematic bacteria. Because of the great potential of plants as a source of antimicrobial drugs, this research planned to compare the in vitro antimicrobial property of some herbal plants to the isolates from the hospital landfill area (Manandhar *et al.*, 2019).

In general, Plant-derived medicines have a number of advantages over synthetic alternatives. They are relatively safer, offer profound therapeutic benefits, and are more affordable. In addition, they are more sustainable and do not leave a harmful footprint on the planet. Green antibiotics, which are produced using renewable resources and minimize the use of synthetic chemicals, are a promising new area of research that has the potential to revolutionize the way we treat infectious diseases.

LITERATURE REVIEW

1.1. Importance of Medicinal Plants: Medicinal plants are those plants which have a great importance because of their ability to treat various diseases. They have bioactive compounds which posses various therapeutic properties such as antibacterial, antifungal, anticancer, anti-inflammatory, antiviral, antiulcer, antidiabetic etc. Kumari *et al.*, 2021 investigates the traditional uses of *Cissampelos pareira* which is a well known medicinal climber plant. They found that approximately 54 phyto molecules have been isolated and characterized from *C. pareira* and the crude extracts of *C. pareira* shows a large number of pharmacological activities such as antimicrobial, antioxidant, antivenom, anticancer, antifertility, antidiabetic, antiulcer, antipyretic etc. They have gained prominence in the Ayurvedic field because they are organic, have no side effects for the consumer and have a wide range of uses. One of them is antioxidant, which is very useful because it protects cells from the free radicals produced inside body by exposure to radiation and smoke. A.A. *et al.*, 2020 in their research 85 different traditional plants were tested for the presence of phytochemical content including alkaloids, glycosides, flavonoids, tannins, saponins and resins. Most commonly found compound among medicinal plants were glycosides (82%; 70 species), tannins (68%; 58 species), alkaloids (56%; 48 species), saponins (52%; 44 species) and flavonoids (35%; 30 species). On the other hand, the least commonly distributed compounds were resins (31%; 26 species). Plants produce secondary metabolites that make them competitive in their own environment. These small molecules exert a wide range of effects on the plant itself and on other living organisms. Phytochemicals including phenolic compounds, flavonoids are of major demand in pharmaceutical, cosmetic as well as food industries (Uysal *et al.*, 2017). Hussain et *al.*, 2018 explains the phytochemicals as the most important driver of the pharmacological effect of medicinal plants. He states that the use of herbal medicines should be based on extensive phytochemical studies to determine the chemical constituents of the herbs involved. From this it is possible to derive knowledge of the resulting pharmacological and toxicological effects, as well as the possible synergistic or antagonistic effects due to the use of multi-component herbal formulas. Hossain *et al.*, 2015 investigated the antioxidant activity in vitro, the total phenol content and the flavonoid concentration of four different extracts from *Adina cordifolia* (Roxb.)

were determined using spectrophotometric methods. The antioxidant activity of the extracts was expressed as percentage inhibition of DPPH radicals and as IC50 values (g/ml). IC50 values ranged from 20.39-38.96 µg/ml. The total phenolic content ranged from 17.48-20.83 mg/g of dry weight of extract, expressed as gallic acid equivalents. The total flavonoid concentrations varied from 17.49 to 22.48 mg/g, expressed as quercetin equivalents. The significant linear correlation was confirmed between the values for the total phenolic content and antioxidant activity of plant extracts. *Adina cordifolia* (Roxb.) can be regarded as promising candidates for natural plant sources of antioxidants with high value.

1.2. Antimicrobial Activity of Plant Extract: Antimicrobial activity refers to the ability of the aqueous or alcoholic extracts of the selected plants to inhibit the growth of the tested microorganisms. T.A. Abere *et al.,* 2007 explains *in-vitro* antimicrobial activity of the extract of *Mitracarpus scarber* leaves. They formulated the crude extract of the leaves of *Mitracarpus scarber* Zucc. as a syrup. The antimicrobial activity of the formulation was assessed using agar plates and concentrations of the extract varying between 25mg/ml and 300mg/ml to determine the minimum inhibitory concentration at 37°c against bacterial and fungal organisms namely, *Escherichia coli*, *Pseudomonas aeruginosa.* The growth of E. coli was inhibited by the formulation at a minimum inhibitory concentration of 75mg/ml. Pseudomonas aeruginosa resist all the concentrations of the formulation used. The presence of sucrose in the formulation rendered the formulation pleasant to taste. The extract from the leaves of Mitracarpus scaber can be formulated into a pleasantly tasting oral dosage form despite its bitter taste. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties. Savitha *et al.*, 2013 studied the antibacterial effect of leaves, stem and root of *Acalypha indica* L. It was evaluated on bacterial strains like *Staphylococcus aureus* and *Salmonella typhi.* The solvent used for the extraction of plants were water and methanol. The *in-vitro* antibacterial activity was performed by agar cup method. The most susceptible bacteria were *Staphylococcus aureus.* The significant antibacterial activity of active extract was compared with the standard antibiotic streptomycin. Panghal *et al.*, 2011 investigate the *Invitro* antimicrobial activity of ten medicinal plants against clinical isolates of oral cancer cases. Results found that seeds extract of *A. tenuifolius*, the tuber extracts

of *A. racemosus*, fruit extract of *B. aegyptiaca*, leaves extract of *T. foenum- graecum*, showed antibacterial activity against most of the isolated microbes from the oral cancer cases. The significant antifungal activity was exhibited by different extracts of *A. racemosus* against *C. albicans* but in contrast to that no significant antifungal activity was observed against *A. fumigatus*. Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism. These compounds have significant therapeutic application against human pathogens including bacteria, fungi or virus. Various researches have been conducted with the extracts of various plants by screening antimicrobial activity and also for the discovery of new antimicrobial compounds and this is the main reason medicinal plants are finding their way into pharmaceuticals. Sen *et al.*, 2012 revealed that Petroleum ether extract shows moderated and aqueous extract shows minimum antimicrobial activity. The effect of different alcoholic extracts viz. ethanol and methanol for antimicrobial activity observed that this difference in the activity is due to the difference between extract compounds in this two extract, the petroleum ether extract of plant *Memecylon umbellatum Burm. F* shows significant antimicrobial activity. Furthermore, water extract from leaves of *P. acerifolium* had been reported to have prominent antimicrobial activity against many gram positive and gram negative pathogenic bacteria. Kamal dev *et al.*, 2014 analyze the antibacterial and antifungal properties of traditional medicinal plants of Shimla and Solan, Himachal Pradesh. Analysis of the mode of antimicrobial action revealed that out of 48 extracts of 41 medicinal plants 50% extracts were bactericidal only 30% plant extracts were fungicidal.

1.3. List of Selected Herbs: Five local plants are selected from Hamirpur district to study their antimicrobial potential on the isolates from five hospital landfills. Plant extracts are used for in vitro antimicrobial screening and their synergistic effect can also be examined in this research.

Table No.1:- List of the Selected Five Herbs.

Sr. No.	Local name	Common name	Scientific name
1	Patha	Velvet leaf	*Cissampelos pareira*
2	Puth kanda	Chaff flower	*Achyranthens aspera*
3	Phool lakri	Lantana	*Lantana camara*
4	Gazar ghass	Congress grass	*Paerthenium hysterophrus*
5	Kuri	Devil's beggertick	*Bidens pilosa*

1.3.1. ***Cissampelos pareira*:** It is commonly known as patha in India. Patha is a long-lasting perennial climbing herb which belongs to family Menispermaceae. It is also known as Ambastha or Laghu Patha in traditional Indian medicine. To date, there are 37 species around the world, but only *C. pareria* grows in India (Mukerji and Bhandari, 1958).

Table No.2:- Taxonomic Classification of *Cissampelos pareira.*

Sr. No.	Scientific Classification	
1	kingdom	Plantae
2	Division	Magnoliophyta
3	Class	Magnoliopsida
4	Order	Ranunculales
5	Family	Menispermaceae
6	Genus	*Cissampelos*
7	Species	*pareira*

Traditional benefits of Patha are it purifies blood, lowers fever, for snake bites, used for malaria, for asthma, to heal wounds, as an appetizer. It is analyzed that *C. pareira* represents mainly alkaloids (mainly isoquinoline). Various types of isoquinoline are isolated including bisbenzylisoquinoline, benzylisoquinoline. Other non-alkaloid constituents are cyclanine, tetrandrine, magnoflorine and berberine (Kumari S. *et al.*, 2021). Patha contains flavonoids, alkaloids, saponins, tannins, steroid tri-terpenoids, and some essential oils. (Yadav *et al.*, 2013) observed the anti-diabetic activity of aqueoextract of *C. pareira.* The dose of 250 mg/kg and 500mg/kg was given to male albino mice for 14 days and a random blood glucose level and body weight was observed periodically. Significant ($p<0.001$) changes were observed in random blood glucose levels in mice after the 14-day treatment period at 500 mg/kg CPAE treatment. Aspartic transaminase, alanine transaminase, alkaline phosphatase, total bilirubin, triglycerides, and creatinine levels were significantly ($p<0.05$) decreased, while glycogen and serum protein levels in liver tissue were significantly ($p<0.05$) increased after CPAE administration. No significant changes in body weight and organ coefficient were observed.

(Patil *et al.*, 2023) studied the in silico screening of alkaloids from *C. pareira* against nine therapeutic targets of malaria. Among them, hayatinin and curin were observed to exhibit strong binding for three antimalarial targets, PfPtS, PfDHFR and PfcGDPK. They also study the molecular dynamics of these top two molecules. The results of this research suggested that this might affect the translation of the Plasmodium parasite to show antimalarial efficacy. *Cissampelos pareira* is also traditionally used to treat snake bites (Giovannini *et al.*, 2017). It could be beneficial for the heart due to the presence of flavonoids and alkanoids. Extracts of *C. pareira* are observed to exhibit many pharmacological activities including antipyretic, anti-inflammatory, anti-arthritic, anti-ulcer, anti-cancer, anti-fertile, anti-microbial, antioxidant and immunomodulatory effects, etc. (Kumari S. *et al.*, 2021).

1.3.2. ***Achyranthens aspera*:** Puth kanda is a weed present throughout India and is considered as important medicinal herb. It predominantly grows like a weed on roadsides. It belongs to family Amaranthaceae.

Table No.3:- Taxonomic Classification of *Achyranthens aspera.*

Sr. No.	Scientific Classification	
1	kingdom	Plantae
2	Division	Magnoliophyta
3	Class	Magnoliopsida
4	Order	Caryophllales
5	Family	Amaranthaceae
6	Genus	*Archyranthes*
7	Species	*aspera*

All of its parts seeds, roots, shoots are used in traditional medicine (S. Hasan, 2014). It is an erect stiff plant which often has a woody stem as a base. Flowers of *A. aspera* have long axillary spikes flowering throughout the year. It has a great importance in ayurvedic, homeopathic and home remedies (Dale *et al.,* 2013). Seeds or flowering spikes are grounded with water and the paste is used for external applications to cure scorpions, snake and reptiles poison also used in night blindness and cutaneous diseases (S. Srivastav *et al.,* 2011).

In Himachal its local name is Puth kanda because of its spikes which are downwards. It has various therapeutic constituents which are used to treat cough, skin rash, antifertility, laxative, antiviral, antitumor, infection, chronic malaria, piles and snake bites, rheumatic pains, iches and skin eruption, diarrhea (London kar *et al.,* 2011). Extract of *A. aspera* contains alkaloids, flavonoids, saponins, steroids and tepenoids. Flavonoids have been observed to slow down or prevent some cancers (Naryana *et al.,* 2001) and also act as anti-inflammatory and antioxidant agents. Chemical constituents like ecdysterone, achyranthine, betaine, pentatriaontane, 6-pentatriacontanone, hexatriacontane and tritriacontane have some pharmacological activities like spermicidal, anti-allergic, analgesic, antipyretic and hypoglyceamic (S. Srivastav *et al.,* 2011).

1.3.3. ***Lantana camara*:** It is an evergreen shrub found in hot temperatures in well drained soil. It has beautiful multicolored blooms which reduce in too much water and fertilizer. The native range of *Lantana camara* is topical and sub-tropical countries worldwide. It belongs to family verbenaceae. Locally it is also known as phool lakri.

Table No.4:- Taxonomic Classification of *Lantana camara*.

Sr. No.	Scientific Classification	
1	kingdom	Plantae
2	Division	Magnoliophyta
3	Class	Magnoliopsida
4	Order	Lamiales
5	Family	Verbenaceae
6	Genus	*Lantana*
7	Species	*camara*

It is an invasive species arrived in India as a decorative shrub in British colonial period. It grows fast and is profuse bloomer and produces blackish little bead like fruits which is considered to have toxin in it. It is perennial and can grow up to ten feet, this shrub is very tough and can climb over trees and entangle with other native plants. It has very important medicinal role in traditional medicine. Extract of *L. camara* constituent's phytochemicals like phenolic, gallic acid and flavonoids (Rabia Naz *et al.*, 2013).

(De melo *et al.*, 2010) in his study analyzes the methanolic extracts for anti proliferative activity against HEp-2 (laryngeal cancer) and NCI-H292 (lung cancer) and *L. camara* shows (25.8±0.19 in NCI-H292).

Abdulla *et al* 2009 analyzes the ethanolic extract of *L. camara* leaves applied on adult male wistar albino to check its wound healing potential. A thin layer of blank placebo is applied to group1 while group2 and group3 are animals dressed with 5% and 10%

extract + placebo. It was observed that group2 and 3 accelerate wound healing while 10% have more healing activity than 5%. So this study shows the wound healing adbility of *L. camara* in animals but more research is required to study the effect of these axtracts on human cells. *L. camara* have antibacterial activity, hemolytic activity, antifungal activity, anti-perglycemic activity, anti-inflammatory activity, mosquito controlling activity (Reddy NM *et al.*, 2013).

1.3.4. *Parthenium hysterophorus*: It is also known as carrot grass because of its leaves which are alike leaves of carrot. It is native to North and South America. It belongs to family Asteraceae. In Himachal it is also known as Gazar ghass.

Table No.5:- Taxonomic Classification of *Parthenium hysterophorus*.

Sr. No.	Scientific Classification	
1	kingdom	Plantae
2	Division	Magnoliophyta
3	Class	Magnoliopsida
4	Order	Asterales
5	Family	Asteraceae
6	Genus	*Parthenium*
7	Species	*hysterophorus*

It is a fast-maturing shrub and it propagate through roots and stem, even a small piece of stem is enough to turn into a new plant. It is an invasive species in India, it enters with imported wheat and since then it created havoc because it has fast germination, better survival ability and nutrient absorption which affect its nearby plants and shows a negative impact on neighboring plants and competitiveness with economical important crops (Lalita *et al.*, 2018).

So for farmers it is a problematic weed as it secretes various biochemicals which affect its neighboring plants and animals. These chemicals inhibit metabolic functioning of nearby plants. It produces a toxin called 'parthenin' it causes respiratory problem in grazing animals and also responsible to bitter taste in milk. Its pollen has ability to cause respiratory allergy like asthma n humans.

P. hysterophorus is considered as traditional medicine throughout Central America. It is used to treat various diseases like Skin infections, amoebic dysentery, and dermatitis. The extracts of carrot grass have phytochemicals which show proper therapeutic properties like larvicidal, antimicrobial, insecticidal, anti-inflammatory, antioxidant and anticancer (kaur L. *et al.*, 2021). *P. hysterophorus* contains various chemicals such as sesquitorpene, lactones, pseudoguainolides, flavonoids, phenolic acids, volatile oils, alkaloids, lipids, steroids etc (Saini *et al.*, 2014).

1.3.5. ***Bidens pilosa*:** It is also known as devil's beggar ticks. It belongs to family Asteraceae. In local areas it is commonly known as kuri. It is an annual plant and widely distributed across temperate and tropical regions. Leaves are oppositely arranged and it has yellow or white flowers seeds are long narrow and ribbed.

It is an edible herb and traditionally has medicinal use. Secondary metabolites such as phenolic acids, terpenes, pheophytins, fatty acids, phytosterols, flavonoids, polyacetylenes, polyacetylene glycosides and phytosterols are isolated from this plant (Tran and Tran, 2016). Polyacetylene glucoside and phenyl propanoid shows anti-inflamatory avtivity (Le. J. *et al.*, 2015).

Table No.6:- Taxonomic Classification of *Bidens pilosa.*

Sr. No.	Scientific Classification	
1	kingdom	Plantae
2	Division	Magnoliophyta
3	Class	Magnoliopsida
4	Order	Asterales

5	Family	Asteraceae
6	Genus	*Bidens*
7	Species	*pilosa*

B. pilosa is used as an herb or ingredient in the herbal tea and herbal medicines. 201 phytochemicals are found due to which they posses anticancer, anti-inflammatory, antioxidant and other bioactivities (Bartolome *et al.*, 2013). Methanol extract and its different subfractions of *B. frondosa* show a great antibacterial activity against *S. aureus*, *P. aeruginosa*, *S. enteritidis*, *B. subtilis* (A. Rahman *et al.*, 2011).

The petroleum extract of *B. pilosa* is tested for its cytotoxic activity for human lung cells. The oral dosages 90, 180 and 360 mg/kg were given to mice and inhibitory rates were 24.76%, 35.85%, and 53.07% respectively. And the extract of petroleum ether shows antiproliferation for human cancer cell lines A549 cells with an IC_{50} of 49.11±2.72 µg/ml (Shen *et al.*, 2018).

RESEARCH GAP IDENTIFICATION

Based upon the literature study a research gap is identified which is, in Himachal there are many areas for research which are unexplored for example there are many unexplored areas and plants present which can be further explored. We can study the antimicrobial potential of local plants and their synergistic effect on the dumping sites of hospitals.

Numerous studies have investigated the synergistic effects in various areas, such as patient isolates, air, and water. However, there remains a significant research gap in the insufficient exploration of the soil in proximity to hospitals, particularly in the Hamirpur district of Himachal Pradesh. This presents an opportunity to conduct a comprehensive study focusing on the hospital's nearby soil, specifically in Hamirpur district, to assess the potential synergistic effects and antimicrobial properties of local plants. By addressing this research gap, a more holistic understanding of the antimicrobial potential within the hospital environment can be achieved.

OBJECTIVES

The main aim of this study is to investigate the effect of the traditional local herbs on such an area which is unexplored yet for research purpose. I will accomplish this aim by meeting the following objectives:

1. Collection, isolation and biochemical characterization of bacterial isolates from the selected hospitals sites.
2. Collection, identification and extract preparation of selected herbs.
3. Evaluation of antimicrobial potential of selected herbs against selected isolates.
4. Assessment of synergistic potential of selected herbs against selected isolates.

PROPOSED METHODOLOGY

For the proposed research problem following Methods will be used.

1. Sample Collection and Pretreatment of Soil Sample (Sharma *et al.*, 2011) :

1. Collection of soil samples using a sterile sampling tool (such as a soil corer or sterile spatula) from 5 random hospital's nearby area of different tehsils in district Hamirpur, which is situated in Himachal Pradesh, India.
2. Collect multiple samples from different areas within the site to capture the microbial diversity.
3. Grind the soil sample using a sterile object and filter it to remove the impurities present in it.

2. Isolation of Microorganisms from the Soil Sample.

A. Serial Dilution: (Sharma *et al.*, 2011)

1. Transfer a small amount of the soil sample (usually 1 gram) to a sterile container (e.g., a test tube or a bottle) containing a known volume of sterile diluent (such as saline or sterile distilled water).
2. Shake the container vigorously to mix the soil with the diluent.
3. Perform a series of dilutions by transferring 1 mL of the soil-diluent mixture to a new container with fresh 9ml of diluent and mix thoroughly. Repeat this process for multiple dilutions.

B. Inoculation and Incubation: (Sharma *et al.*, 2011)

1. Take 100 µL of each diluted sample and spread it evenly on the surface of Nutrient Agar. Use a sterile spreader or a glass rod to distribute the sample over the agar surface. Repeat the plating process for each dilution.
2. Incubate the agar plates upside down at the appropriate temperature (usually 25-37°C) for a suitable incubation period (typically 24-48 hours). The incubation conditions can be adjusted to favor the growth of specific bacteria if desired.

3. Identification of Isolates by using Physical and Biochemical Methods.

a) **Identify Grown Colonies on the Basis of their Morphology** like color, size, shape, texture, elevation, surface, edges, structure, degree of growth, nature (bergey's manual, 1994).

b) The Mixed Culture of Isolates can be Identifying by using Staining Methods (Siguenza *et al.*, 2019).

1. **Gram Staining:** Prepare a heat-fixed smear of the bacteria on a glass slide. Flood the the smear with crystal violet stain and let it sit for 1 minute after that rinse the slide gently with water. Flood the slide with iodine solution and let it sit for 1 minute, rinse the slide gently with water. Decolorize the slide by flooding it with ethanol or acetone and quickly rinsing with water and then counterstain the slide with safranin for 1 minute. Rinse the slide gently with water. Blot the slide dry and observe under a microscope. Gram-positive bacteria will appear purple, while gram-negative bacteria will appear pink.
2. **Endospore Staining:** Take a dry glass slide and prepare a thin smear with the help of sterile inoculating loop. Allow it to air dry and fix it with gentle heat. Flood the slide with 5% Malachite green solution. Allow the stain to be in contact with the smear for 2-3 minutes, heat the preparation for 5-6 minutes, and then cool. Wash the slide with slowly running tap water and then counterstain the smear with 0.5% safranin for 30 seconds. Wash the slide with distilled water and observe under the microscope.

c) Select Representative Colonies with Different Morphologies (size, shape, color) and transfer them to separate agar plates to obtain pure cultures (Bonnet *et al* 2019).

Streak the selected colonies onto fresh agar plates using a quadrant streaking technique or other appropriate methods to isolate individual bacterial colonies. Different selective media are used to grow a selective type of microorganism like:

a. **MacConkey Agar:** This medium contains bile salts and crystal violet, which inhibit the growth of Gram-positive bacteria while allowing the growth of Gram-negative bacteria. It is commonly used to isolate and differentiate lactose-fermenting (pink colonies) from non-lactose-fermenting (colorless colonies) Gram-negative bacteria, such as members of the Enterobacteriaceae family.
b. **Mannitol Salt Agar (MSA):** MSA contains a high concentration of salt (7.5%), making it selective for the growth of halophilic bacteria, particularly *Staphylococcus* species. It also contains mannitol and the pH indicator phenol red, allowing the differentiation of mannitol-fermenting (yellow colonies) from non-fermenting (red colonies) *Staphylococci*.

c. **Eosin Methylene Blue (EMB) Agar:** EMB agar is selective for Gram-negative bacteria, especially members of the Enterobacteriaceae family. It contains dyes eosin Y and methylene blue, which inhibit the growth of Gram-positive bacteria. It can differentiate lactose-fermenting (dark-centered colonies) from non-lactose-fermenting (colorless colonies) bacteria based on their ability to ferment lactose.

d. **Sabouraud Agar:** This medium is selective for fungi and inhibits the growth of bacteria. It contains a low pH (typically around 5.6) and the antibiotic chloramphenicol to suppress bacterial growth. Sabouraud agar is commonly used for the isolation and cultivation of dermatophytes and yeasts.

e. **Cetrimide Agar**: This medium is selective for the isolation and identification of *Pseudomonas aeruginosa*. It contains the detergent cetrimide, which inhibits the growth of most other bacteria, allowing the growth of *Pseudomonas* species.

d) **Different methods can be used for Biochemical Characterizations which are given below (Talaiekhozani, 2013).**

a. **Catalase Test**: Used for various bacterial species to differentiate between catalase-positive organisms, such as *Staphylococcus* and *Micrococcus*, and catalase-negative organisms like *Streptococcus*.

b. **Oxidase Test:** Primarily used for the identification of bacteria that possess cytochrome oxidase, including many Gram-negative bacteria like *Pseudomonas*, *Neisseria*, and *Vibrio* species.

c. **Coagulase Test:** Specifically used for the identification of *Staphylococcus aureus*, a pathogenic bacterium known for causing various infections in humans.

d. **Indole Test:** Generally performed on Enterobacteriaceae family members, including *Escherichia coli* and *Klebsiella pneumoniae*, to determine their ability to produce indole.

e. **Methyl Red/Voges-Proskauer (MR-VP) Test:** Performed on various Enterobacteriaceae species, with Methyl Red indicating the production of mixed acids (e.g., *Escherichia coli*), and Voges-Proskauer detecting the production of acetoin (e.g., *Enterobacter aerogenes*).

f. **Citrate Utilization Test:** Frequently used for the identification of Enterobacteriaceae, specifically to determine their ability to utilize citrate as a carbon source.

4. **Collection of Selected Herbs from the Hamirpur District of Himachal Pradesh.**
Puth kanda, patha, phool lakri, gazar ghass, kuri, are the local herbs collected from the random areas of Hamirpur district for research study.

5. **Preparation of Leaves Extract (Zgoda and Porter, 2001).**
   a. Pretreatment of leaves: wash them under running water then air dry them for a month in room temperature.
   b. Grind them separately to make a fine powder out of them.
   c. Take 60gm of fine powder and dissolve it in 160ml of 70% of ethanol at room temperature for 3 successive days.
   d. Then filter the mixture and extracts are concentrated on hot plate and heated at 30-40°c.
   e. Re dissolve the plant extract in 2.5% dimethyl sulfoxide (DMSO) which is maximum volume of DMSO that could be used to dissolve solid extracts The solvent DMSO (2.5%) that would not inhibit growth of the microorganisms will be used as the negative control for all the experiments.
6. **Antimicrobial Activity of Each Herb on Selected Isolates (Lihan *et al.*2014).**
   1 Antimicrobial assay of extracts of different herbs are performed by agar well diffusion method in Mueller Hinton Agar (MHA) plates.
   2 Prepare nutrient agar plates and sterilize them to provide a suitable growth medium. Then inoculate the plates with isolated microorganism ensuring even distribution on the agar surface. Using a sterile pipette tip, create 7 wells in the agar, keeping them adequately spaced apart. Carefully add different dilutions of the plant extract into the wells. Allow the plates to stand briefly to facilitate diffusion. Then, incubate the plates at the appropriate temperature and duration for the microorganisms being tested. After the incubation period, observe the plates for the presence of zones of inhibition clear areas around the wells where the test substances have hindered the growth of microorganisms. Measure the diameter of the inhibition zones using a ruler or calipers, recording the results along with any relevant observations.

7. **Synergistic Potential of the Best Plant Extract:**
Evaluation of plant which shows best antimicrobial potential against isolated microorganisms and its synergistic effect on the selected microorganisms which we

isolate from the selected soil samples. To check synergistic potential mix all the plant extract then check its antimicrobial activity on isolated microbes by agar well diffusion and compare its result with the synergistic potential of the best antimicrobial herb and antibiotic.

8. **Molecular Characterization of Selected Isolates:** (Bouchet et al., 2008)

For the molecular characterization of isolated strains 16s rRNA sequencing is used. Following steps will be used for molecular characterization of selected isolates of microorganisms.

1. Isolation of DNA
2. Polymerase Chain reaction
3. Agarose gel electrophoresis
4. Elution of DNA
5. Restriction digestion
6. Southern blotting
7. Autoradiography

9. The obtained results will be analyzed and published in research thesis.

THESIS OUTLINE

Chapter 1 (Introduction): This chapter includes the general information regarding traditional medicinal plants, microflora present in the soil and importance of using antibacterial products of medicinal plants over chemicals.

Chapter 2 (Literature review): It includes the deep review of the literature of various researchers regarding antimicrobial and pharmaceutical properties of medicinal plants. This chapter also includes the research gap indentified through literature study and objectives of the study.

Chapter 3 (Materials and Methods): This chapter contains the research methodology. Methodology of sample collection, isolation and characterization, Preparation of plant extract, Antimicrobial activity of plant extract against selected isolates, Synergistic potential and molecular characterization.

Chapter 4 (Result and Discussion): This chapter will describe the results of isolation and characterization of isolated microorganism, antimicrobial activity of plant extract on the selected isolates and molecular characterization.

Chapter 5 (Summary and Conclusion): This chapter will comprise the conclusion of the whole study based on objectives along with summary. The scope of future research will also be discussed.

Chapter 6 (References): This chapter will include the list of sources that are used in research study.

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CERTIFICATE
(SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Synopsis for Ph.D. Thesis titled, **"Evaluation of synergistic and antimicrobial potential of selected herbs against the isolates of nearby area of hospitals from Hamirpur, Himachal Pradesh."**, submitted by **Ms. Niharika Sharma** to the Career Point University, Hamirpur (H.P.), India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in the Division of Microbiology, School of Pharmaceutical and health sciences has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated 8th June 2023.

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